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Emergence of Linezolid-Resistant *Staphylococcus aureus* after Prolonged Treatment of Cystic Fibrosis Patients in Cleveland, Ohio▽

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Linezolid (LZD)-resistant *Staphylococcus aureus* (LRSA) isolates were monitored from 2000 to 2009 in Cleveland, OH. LRSA first emerged in 2004 only in cystic fibrosis (CF) patients, with 11 LRSA-infected CF patients being identified by 2009. LRSA was isolated from 8 of 77 CF patients with *S. aureus* respiratory tract infection treated with LZD from 2000 to 2006. Analysis of clinical data showed that the 8 CF patients with LRSA received more LZD courses (18.8 versus 5.9; $P = 0.001$) for a longer duration (546.5 versus 211.9 days; $P < 0.001$) and had extended periods of exposure to LZD (83.1 versus 30.1 days/year; $P < 0.001$) than the 69 with LZD-susceptible isolates. Five LRSA isolates included in the clinical analysis (2000 to 2006) and three collected in 2009 were available for molecular studies. Genotyping by repetitive extrapalindromic PCR and pulsed-field gel electrophoresis revealed that seven of these eight LRSA strains from unique patients were genetically similar. By multilocus sequence typing, all LRSA isolates were included in clonal complex 5 (seven of sequence type 5 [ST5] and one of ST1788, a new single-locus variant of ST5). However, seven different variants were identified by *spa* typing. According to the *Escherichia coli* numbering system, seven LRSA isolates contained a G2576T mutation (G2603T, *S. aureus* numbering) in one to four of the five copies of domain V of the 23S rRNA genes. One strain also contained a mutation (C2461T, *E. coli* numbering) not previously reported. Two strains, including one without domain V mutations, possessed single amino acid substitutions (Gly152Asp or Gly139Arg) in the ribosomal protein L3 of the peptidyltransferase center, substitutions not previously reported in clinical isolates. Emergence of LRSA is a serious concern for CF patients who undergo prolonged courses of LZD therapy.

Staphylococcus aureus is a common pathogen in cystic fibrosis (CF) lung disease (33). The prevalence of methicillin-resistant *S. aureus* (MRSA) in CF patients is increasing, with 20% of patients reported to have been harboring this organism in 2008 (8, 33). Persistent MRSA infection in CF patients has been associated with a more rapid decline in lung function and survival (9–11). Since the most common cause of death in CF patients continues to be respiratory failure, treatment of respiratory tract infections (RTIs), including those due to MRSA, is important (33, 41).

Linezolid (LZD) has become an attractive alternative to vancomycin in the treatment of RTIs due to MRSA, especially in non-CF patients. LZD achieves high concentrations in lung tissue, epithelial lining fluid, and bronchial mucosa (1, 32, 41). Furthermore, the availability of oral dosing may minimize the need for hospital admissions (14, 39). LZD inhibits bacterial protein synthesis through binding to the peptidyltransferase center (PTC) of the 50S ribosomal subunit (26).

LZD resistance in *S. aureus* is uncommon, with >99% of isolates being susceptible in recent surveys (20, 35). To date, the following mechanisms responsible for LZD resistance have been reported in clinical isolates of *S. aureus*: (i) mutations in the domain V region of one or more of the five or six copies of the 23S rRNA gene (e.g., G2576T, T2500A, and G2447T, *Escherichia coli* numbering system) (24, 31), (ii) acquisition of the plasmid-mediated ribosomal methyltransferase *cfr* gene (20, 25, 28, 36, 43), and (iii) deletions or mutations in the ribosomal protein L3 of the PTC (22, 23). Additional mutations in domain V of the 23S rRNA genes and substitutions in ribosomal protein L4 of the PTC are also reported in laboratory-derived LZD-resistant *S. aureus* (LRSA) strains (23). Although LRSA isolates are emerging among patients undergo-

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ing prolonged periods of therapy (20, 24, 30, 44, 47), only three cases, one in Brazil and two in England, of infection due to resistant strains have been reported in CF patients. Both LRSA isolates identified in England were sequence type 36 (ST36), a widespread MRSA type observed in United Kingdom hospitals, whereas the type of the strain from Brazil is unknown (15, 19).

In the present work, we describe the emergence of LRSA in our institution over a 10-year period and analyze the clinical characteristics of 77 CF patients who received LZD treatment for *S. aureus* RTIs, including those with LRSA isolates. To elucidate the mechanism(s) responsible for this phenotype, molecular analyses of LRSA isolates were performed, as well as analysis of virulence factors and clonality. Our results show that the emergence of LZD resistance in *S. aureus* is a serious concern for CF patients undergoing prolonged courses of therapy.

MATERIALS AND METHODS

Study design and subjects. For this analysis we used data from the patient registry at the CF Center at Rainbow Babies and Children's Hospital (CFC-RBCH; Cleveland, OH). The registry contains comprehensive demographic, clinical, and microbiological information on all CF patients seen at the center, and this information is entered into a database using a standardized form. Antimicrobial susceptibility data for *S. aureus* isolates collected from all patients during the years from 2000 to 2009 were also extracted from laboratory records.

The clinical characteristics of CF patients with *S. aureus* RTIs treated with LZD over a 7-year period (2000 to 2006) were analyzed. Patients were included in the study if they met the following criteria: (i) evaluated at the CFC-RBCH from January 2000 to December 2006, (ii) received LZD treatment during this time period, and (iii) had available sputum and/or throat swab specimen culture results, including susceptibility data regarding LZD. The design was a cohort study comparing patients who had cultures of samples from the respiratory tract with LRSA with those who had cultures of samples with LZD-susceptible *S. aureus* (LSSA) isolates. Demographic, microbiologic, and clinical data were collected for analysis and included age, gender, weight, ethnicity, CF transmembrane conductance regulator mutation, dose and length of LZD therapy, respiratory culture and antimicrobial susceptibility test (AST) data, concurrent antibiotic use, and number of hospital admissions. This study was approved by the Institutional Review Board for Human Subjects Investigation of the University Hospitals Case Medical Center (UHCMC).

Clinical isolates. AST data and species identification were routinely assessed using the MicroScan system (Siemens Healthcare Diagnostics). MICs for LZD-nonsusceptible isolates were then determined by microdilution in Mueller-Hinton broth (BBL, Becton Dickinson). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria. LRSA isolates were defined as those with MICs of ≥ 8 μg/ml for LZD (7).

Statistical analysis. A descriptive analysis of CF patients was performed with calculation of means, standard deviations, and medians for continuous variables in each cohort. Categorical variables were measured using proportions. Bivariate analyses were conducted for continuous variables using the Wilcoxon rank-sum test, and categorical variables were compared using Fisher's exact test. Since differences between groups could be confounded by including data collected after the first LRSA-positive culture, analyses that included only antibiotic and hospitalization data for the LRSA group in the years prior to the first LRSA-positive culture were performed. We did not conduct *a priori* statistical power or sample size calculations because (i) we used previously collected data from our CF patient registry, (ii) the prevalence of LRSA isolates in the United States is low (<0.1%) (20, 35), and (iii) only a small number of patients have received LZD at our institution (i.e., 82 of 390 CF patients). Statistical analysis was performed using the STATA program (version 10.0; StataCorp).

Molecular characterization of mechanism(s) responsible for linezolid resistance. Genomic DNA of LRSA isolates was extracted using a MasterPure Gram-positive organism DNA purification kit (Epicenter Biotechnologies). PCR amplification of the surrounding area of the six 23S rRNA domain V genes (i.e., *rrn1* to *rrn6*) was performed using high-fidelity *rTth* DNA polymerase XL (Applied Biosystems). Domain V of *rrn1* to *rrn5* was analyzed as reported by Pillai et al. (31), whereas that of *rrn6* was studied as suggested by Meka et al. (24). These PCR products were purified using a QIAquick PCR purification kit (Qiagen Sciences). PCR amplification of domain V of each of the purified *rrn* DNA fragments was performed as previously reported (31). Genes *rplC*, *rplD*, and *rplV* (encoding ribosomal proteins L3, L4, and L22 of the PTC, respectively) and *cfr* were also analyzed by PCR and DNA sequencing as described previously (21, 22). DNA traces for domain V and *rpl* genes were compared to the deposited complete genome sequence of *S. aureus* N315 (GenBank accession no. NC_002745). Southern blot hybridization was performed with EcoRI-digested genomic DNA as previously described (5) using a specific probe of 420 bp for the domain V region(s) (31).

rep-PCR and PFGE. The genetic relatedness of isolates was investigated by repetitive extrapalindromic PCR (rep-PCR) using the semiautomated DiversiLab system (bioMérieux Inc.) on genomic DNA extracts obtained using an UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Inc.). The clonality of the LRSA isolates was also analyzed by pulsed-field gel electrophoresis (PFGE) using SmaI digests, according to standard methods (42).

Characterization of resistance genes, virulence genes, and MLSTs by PCR/ESI-MS. We employed the T5000 Biosensor system (Ibis Biosciences), a PCR electrospray ionization mass spectrometry (PCR/ESI-MS) platform. A MRSA typing and characterization kit (Ibis Biosciences) was used. The kit detects and analyzes the following genes (with functions in parentheses): *tufB* (identification of species of staphylococci), *nuc* (confirmation of *S. aureus* species), *mecA* (methicillin resistance) (6), *mupA* (high-level mupirocin resistance), *ileS* (low-level mupirocin resistance), *lukD*/*PVL* (detects both the *lukS-PV* gene, encoding a component of the Panton-Valentine leukocidin [PVL] virulence factor, and the *lukD* gene, encoding the γ-hemolysin) (46), the gene for PVL type (a mutation in PVL associated with the R and H genotypes of PVL) (29), and the gene for toxic shock syndrome toxin 1 (TSST-1). Furthermore, the assay uses eight primer pairs to perform multilocus sequence typing (MLST) targeting six housekeeping genes (*aroC*, *aroE*, *gmk*, *pta*, *tpi*, *yqi*). MLSTs were compared to PFGE profiles (e.g., USA types) and clonal complexes as previously reported (17).

MLST and *spa* typing. Standard MLST was performed by analyzing seven housekeeping genes (*aroC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) according to the scheme of Enright et al. (12). DNA sequences were interpreted using the tools provided on www.mlst.net/. *S. aureus* isolates were also typed by analyzing the polymorphic X, or short sequence repeat (SSR), region of the staphylococcal protein A (*spa*) gene (40). DNA sequencing results were interpreted using Ridom StaphType software (Ridom GmbH, Würzburg, Germany) (18).

RESULTS AND DISCUSSION

LRSA isolates, including those from CF patients, were previously thought to be extremely uncommon (20). Since only three sporadic cases have been described in CF patients, data regarding clinical characteristics and risk factors associated with the development of RTIs due to LRSA isolates in this special population are lacking (15, 19). In the present study, we tracked the emergence of LRSA in our institution (CFC-RBCH) and compared the clinical characteristics of CF patients with RTIs due to LRSA with the clinical characteristics of the larger group of those with RTIs due to LSSA isolates. Furthermore, a detailed molecular analysis of the mechanisms associated with LZD resistance, along with genotyping using different methods, was carried out for the LRSA strains.

Clinical isolates and identification of cohort of CF patients with and without LRSA infection. The first LRSA isolate was detected in July 2004, with 40 such isolates being detected by December 2009. All these isolates were recovered from respiratory specimens from 11 CF patients; LRSA isolates were not recovered from non-CF patients (Table 1).

Three hundred ninety CF patients were evaluated at the CFC-RBCH from January 2000 to December 2006. Eighty-two CF patients (21.0%) received at least one course of LZD during this time period. Of these, five patients were excluded from the analysis because the inclusion criteria were not met. Thus, the analysis group consisted of 77 CF patients documented to be harboring *S. aureus* isolates. Eight (10.4%) out of these 77 patients had at least one respiratory specimen culture with a LRSA isolate.

TABLE 1. Linezolid susceptibility of *S. aureus* isolates from CF and other patients, by year

Patient group	Result for LRSA isolates by year[a]						
	2003	2004	2005	2006	2007	2008	2009
No. of CF patients with LRSA/no. of *S. aureus* isolates tested (%)	0/788 (0)	3/306 (1.0)	3/244 (1.2)	8/272 (2.9)	1/257 (0.4)	4/271 (1.5)	21/806 (2.6)
No. of new CF patients acquiring LRSA[b]	0	3	3	2	1	1	1
No. of CF patients with LRSA by yr	0	3	3	5	1	4	7
No. of all other patients with LRSA/no. of *S. aureus* isolates tested	0/2,167	0/2,125	0/2,816	0/3,535	0/2,710	0/3,427	0/3,594

[a] LRSA isolates were not detected from 2000 to 2002.
[b] Year of first detection of LRSA.

Clinical aspects of CF patients with LRSA. Demographic and clinical data for the 77 CF patients, analyzed and categorized by LZD susceptibility, are summarized in Table 2. The eight patients who developed RTIs due to LRSA received more courses of LZD (18.8 versus 5.9 courses; $P = 0.001$) and for a longer duration (546.5 versus 211 days; $P < 0.001$) than those with infections due to LSSA. Furthermore, patients with LRSA had significantly more exposure to LZD than those with LSSA (83.1 versus 30.1 days/year; $P < 0.001$). These findings are consistent with those of the previous reports describing non-CF patients with infections due to LRSA (24, 30, 44, 47) and the information presented for the three sporadic CF cases previously published (15, 19). Other statistically significant differences between the two groups of patients were not observed (Table 2).

In Table 3 we show the descriptive characteristics of the

TABLE 2. Characteristics of CF patients with *S. aureus* respiratory infection who were treated with LZD from 2000 to 2006

Characteristic	All patients ($n = 77$)	Patients with LRSA infection ($n = 8$)	Patients with LSSA infection ($n = 69$)	*P* value[a]
No. (%) patients of female gender	41 (53.3)	4 (50.0)	37 (53.6)	1.0
Mean ± SD age (yr)[b]	14.6 ± 9.6	13.6 ± 9.0	14.8 ± 9.8	0.62
No. (%) of patients with delta F508[c]	45 (58.4)	4 (50.0)	41 (59.4)	0.71
No. (%) of Caucasian patients	74 (96.0)	8 (100)	66 (95.7)	1.0
LZD courses in 7 yr				
Mean ± SD	7.2 ± 8.2	18.8 ± 14.1	5.9 ± 6.1	0.001
Median (interquartile range)	4 (1–11)	15.5 (8–27)	4 (1–8)	
LZD exposure over 7 yr (days)[d]				
Mean ± SD	245.9 ± 331.3	546.5 ± 240.2	211.0 ± 323.8	<0.001
Median (interquartile range)	98 (35–336)	467 (371–691)	74 (28–205)	
LZD exposure/yr in pre-LRSA period (days)[d]				
Mean ± SD	35.6 ± 49.1	83.1 ± 50.0	30.1 ± 46.3	<0.001
Median (interquartile range)	14 (5–45)	75 (44–116)	11 (4–29)	
Additional no. of antibiotics over 7 yr				
Mean ± SD	6.0 ± 9.6	13 ± 20.0	5.2 ± 7.4	0.07
Median (interquartile range)	3 (1–8)	8 (4–9)	2 (1–7)	
Additional no. of antibiotics/yr in pre-LRSA period				
Mean ± SD	0.9 ± 1.7	2.3 ± 4.3	0.7 ± 1.0	0.11
Median (interquartile range)	0.4 (0.1–1.0)	0.8 (0.5–1.7)	0.3 (0.1 ± 1.0)	
No. of hospitalizations in 7 yr				
Mean ± SD	6.4 ± 9.1	12.8 ± 19.6	5.7 ± 6.9	0.18
Median (interquartile range)	3 (1–10)	6 (2.5–12)	3 (1–10)	
No. of hospitalizations/yr in pre-LRSA period				
Mean ± SD	0.9 ± 1.5	2.0 ± 3.7	0.8 ± 1.0	0.38
Median (interquartile range)	0.4 (0.1 ± 1.4)	0.7 (0.2 ± 1.8)	0.4 (0.1 ± 1.4)	
No. (%) of patient deaths	5 (6.5)	1 (12.5)	4 (5.8)	
No. (%) of patient lung transplants	8 (10.4)	2 (25.0)	6 (8.7)	

[a] Comparison of LRSA and LSSA groups using Wilcoxon rank-sum or Fisher's exact test.
[b] Age at the midpoint of the year 2000.
[c] Homozygous.
[d] Cumulative.

TABLE 3. Summary of CF patients with pulmonary infection due to LRSA isolates and treated with LZD from 2000 to 2006[a]

Characteristic	Patient 1 (strain NA)	Patient 2 (strain M36)	Patient 3 (strain I32)	Patient 4 (strain NA)	Patient 5[b] (strain M41)	Patient 6[b] (strain M94)	Patient 7 (strain M86)	Patient 8 (strain NA)
Age (yr)[c]	3 (F)	7 (M)	14 (F)	33 (M)	10 (M)	11 (F)	13 (M)	14 (F)
Mo/yr of first dose of LZD	12/2002	7/2000	12/2001	12/2000	9/2000	2/2001	12/2002	7/2000
Mo/yr when last LSSA isolate detected before occurrence of LRSA	3/2003	1/2004	Never	4/2005	2/2004	11/2003	7/2005	4/2005
Mo/yr when first LRSA isolate detected	10/2004	7/2004	12/2004	6/2005	2/2005	12/2005	12/2006	8/2006
Time between first dose of LZD and first LRSA isolate detected (mo)	22	48	35	54	53	58	48	73
No. of days of LZD use per yr/no. of treatments (dose, time)[d]								
2000	0/0	32/1 (9 mg/kg, q12h)	0/0	10/1[e] (600 mg, q12h)	35/2 (4 mg/kg, q12h)	0/0	0/0	14/1 (600 mg, q12h)
2001	0/0	121/7 (10 mg/kg, q12h)	1/1 (600 mg, q12h)	120/0[e] (600 mg, q12h)	70/4 (4 mg/kg, q12h)	63/4 (4 mg/kg, q12h)	0/0	33/2 (600 mg, q12h)
2002	23/1 (9 mg/kg, q12h)	165/11 (11 mg/kg, q12h)	176/17 (600 mg, q12h)	40/0[e] (600 mg, q12h)	70/2 (10 mg/kg, q12h)	0/0	15/1 (600 mg, q12h)	69/3 (600 mg, q12h)
2003	365/0 (9 mg/kg, q12h)	224/4 (9 mg/kg, q12h)	211/20 (600 mg, q12h)	0/0	185/2 (10 mg/kg, q12h)	21/1 (600 mg, q12h)	28/2 (600 mg, q12h)	144/5 (600 mg, q12h)
2004	309/1 (9 mg/kg, q12h)	44/4 (9 mg/kg, q12h)	116/8 (600 mg, q12h)	100/2 (600 mg, q12h)	117/5 (7 mg/kg, q12h)	99/2 (600 mg, q12h)	32/2 (600 mg, q12h)	14/1 (600 mg, q12h)
2005	53/1 (10 mg/kg, q12h)	56/2 (600 mg, q12h)	0/0	28/2 (600 mg, q12h)	114/5 (9 mg/kg, q12h)	195/4 (600 mg, q12h)	89/3 (600 mg, q12h)	29/1 (600 mg, q12h)
2006	268/1 (9 mg/kg, q12h)	71/2 (600 mg, q12h)	0/0	0/0	77/3 (600 mg, q12h)	0/0	199/7 (600 mg, q12h)	127/3 (600 mg, q12h)
Anti-*Staphylococcus* therapy given after first LRSA-positive culture	TMP-SXT, DOX, VAN	TMP-SXT, DOX, VAN	MEM, CLIN	TMP-SXT, CAZ, VAN	TMP-SXT, DOX	TMP-SXT, DOX	None	None
Outcome[f]	Alive	Alive	Deceased in 2006	Lung transplant in 2006	Alive	Alive	Alive	Lung transplant in 2006

[a] NA, not available for molecular characterization; TMP-SXT, trimethoprim-sulfamethoxazole; DOX, doxycycline; VAN, vancomycin; CLIN, clindamycin; MEM, meropenem; CAZ, ceftazidime; M, male; F, female; q12h, every 12 h.

[b] Patients 5 and 6 are siblings.

[c] Age at the midpoint of the year 2000. The patient sex is given in parentheses.

[d] For years with treatment days but zero treatments, the course was continued from the previous year. All linezolid treatments were administered orally. If more than one treatment regimen was used, then the average dose for the year is given.

[e] Dosed the first 10 days of each month from December 2000 to April 2002.

[f] On 31 December 2006.

TABLE 4. Phenotype and molecular analysis of the mechanism(s) responsible for LZD resistance in LRSA isolates[a]

Isolate, yr of collection (patient no.)	AST[c]	LZD MIC (μg/ml)	Result of molecular analysis of genes conferring LZD resistance									
			Domain V 23S rRNA copies						*cfr*	Peptidyltransferase center		
			rrn1	*rrn2*	*rrn3*	*rrn4*	*rrn5*	*rrn6*		L3 (*rplC gene*)	L4 (*rplD gene*)	L22 (*rplV gene*)
M36, 2004 (patient 2)	AZI (R), CLIN (R), VAN (S), TET (S), TMP-SXT (R), RIF (NT)	>256	G2576T	G2576T	−	−[e]	+	G2576T	−	Gly139Arg	+	+
I32, 2004 (patient 3)	AZI (R), CLIN (I), VAN (S), TET (S), TMP-SXT (S), RIF (S)	>256	G2576T	G2576T	−	G2576T	G2576T	+	−	+	+	+
M41, 2005 (patient 5[d])	AZI (R), CLIN (R), VAN (S), TET (S), TMP-SXT (R), RIF (S)	16	+	+	−	G2576T	+	+	−	+	+	+
M94, 2005 (patient 6[d])	AZI (R), CLIN (I), VAN (S), TET (S), TMP-SXT (R), RIF (S)	64	G2576T	G2576T	−	+	+	G2576T	−	+	+	+
M86, 2006 (patient 7)	AZI (R), CLIN (R), VAN (S), TET (R), TMP-SXT (S), RIF (S)	64	G2576T	+	−	G2576T	G2576T C2461T	+	−	+	+	+
M92, 2009[b]	AZI (R), CLIN (R), VAN (S), TET (S), TMP-SXT (S), RIF (S)	32	+	G2576T	−	G2576T	G2576T	G2576T	−	+	+	+
M32, 2009[b]	AZI (R), CLIN (R), VAN (S), TET (S), TMP-SXT (S), RIF (S)	8	+	+	−	+	+	+	−	Gly152Asp	+	+
M71, 2009[b]	AZI (R), CLIN (R), VAN (S), TET (S), TMP-SXT (S), RIF (S)	32	+	G2576T	−	G2576T	G2576T	+	−	+	+	+

[a] R, resistant; S, susceptible; +, complete gene or protein without mutations; −, gene not amplified by PCR analysis; AZI, azithromycin; CLIN, clindamycin; VAN, vancomycin; TET, tetracycline; TMP-SXT, trimethoprim-sulfamethoxazole; RIF, rifampin.

[b] These isolates were recently collected from CF patients who were not included in the clinical statistical analysis covering the 7-year period (2000 to 2006).

[c] All isolates were resistant to methicillin (MRSA) and were *mecA* positive according to PCR/ESI-MS analysis. Results were interpreted according to CLSI guidelines (7).

[d] Patients 5 and 6 are siblings.

[e] —, the 23S rRNA gene was not amplified by PCR but was possibly present on the basis of the results of Southern blotting (Fig. 1).

eight CF patients with RTIs due to LRSA. A wide range of LZD treatment regimens (all of them administered orally) was recorded. In particular, due to clinical circumstances considered by the treating physicians, the dosing of LZD for the four CF patients <12 years old ranged from 4 mg/kg of body weight to 11 mg/kg and LZD was prescribed every 12 h (the correct LZD dosage should be 10 mg/kg every 8 h [q8h]) (16). As we did not collect data on patient compliance with LZD therapy, which was reported for the first case of a pediatric CF patient with LRSA, compliance with the prescribed regimen is unknown (15). Optimal pharmacokinetic/pharmacodynamic (PK/PD) indices are important for antimicrobial efficacy and for suppression of resistance to LZD. The potential for resistance development appears to be higher when the drug concentration is in the vicinity of the MIC of the isolate (3). In this context, two additional points should be taken into consideration for the remaining four CF patients who were >12 years old and who received the recommended dosage of 600 mg every 12 h (Table 3) (16). First, sputum levels of LZD in adult CF patients 12 h after a 600-mg dose are 3.6 ± 2.1 μg/ml (38), a concentration close to the breakpoint of susceptibility (i.e., ≤4 μg/ml) and the MIC_{90} (i.e., 2 μg/ml) of *S. aureus* isolates (7, 13). Second, since CF patients have enhanced hepatic and renal clearance (34), treatment with the suggested LZD dosage may not achieve the ideal PK/PD ratio of the area under the concentration-time curve/MIC of >80 (4, 37).

Except for patients 5 and 6, who are siblings, contact among patients with LRSA was not noted on the basis of hospital admissions or outpatient visits. With regard to the outcome, five of the patients were alive during the period of the study. Two patients underwent lung transplantation (recurrence of LRSA is not documented), and one patient died. Unfortunately, our study did not include a sufficient number of patients to determine any consequence of LZD resistance on clinical outcome. In future studies, the importance of this and other resistance phenotypes, as well as the interplay of various virulence factors, will need to be evaluated.

Mechanisms of resistance to LZD. The first LRSA isolate collected from eight CF patients, including five of the eight LRSA isolates identified during the clinical study (2000 to 2006) and the three collected in 2009, was available for molecular analysis. These isolates express different levels of resistance to LZD, with MICs ranging from 8 to >256 μg/ml (Table 4). With the exception of isolate M92, Southern blotting demonstrated the presence of five domain V copies of the 23S rRNA *rrn* genes in the LRSA strains; M92 has six copies (Fig. 1). Sequence analysis reveals that all isolates contained *rrn1*, *rrn2*, *rrn5*, and *rrn6*, with *rrn4* being present in all but one isolate (M36); *rrn3* was absent from all isolates (Table 4). As previously observed (2), the number of domain V copies with a mutation is directly proportional to the increase in the MIC for LZD. In particular, one to four copies of the *rrn* genes of seven of the eight LRSA isolates contained a G2576T mutation according to the *E. coli* numbering system (G2603T according to the *S. aureus* numbering system) in six different combinations, with only two strains sharing a common pattern. We also note that isolate M86 possessed another mutation (i.e., C2461T, according to the *E. coli* numbering system) that was not previously reported.

None of the LRSA isolates possessed the *cfr* gene, whereas

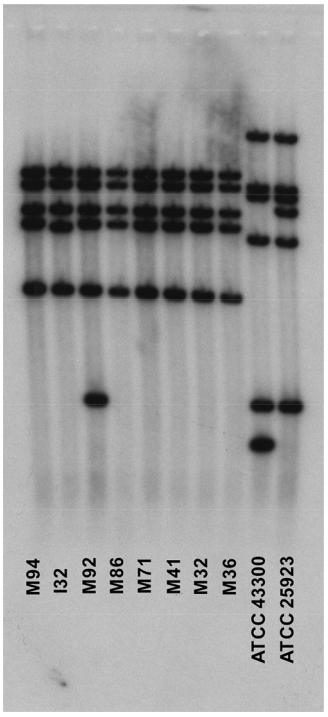

FIG. 1. Southern blot hybridization using the domain V probe for LRSA isolates (31). Genomic DNA was digested with EcoRI. Two *S. aureus* control strains (ATCC 43300 and ATCC 25923) were used. Seven out of eight LRSA isolates possess five copies of the domain V 23S rRNA gene, supporting in part the results of the molecular analysis (Table 4). Only one LRSA isolate (M92) has six 23S rRNA copies. Isolates M92, M32, and M71 were found in 2009 and were not included in the clinical statistical analysis covering the 7-year period (2000 to 2006).

two strains had single and unrelated amino acid substitutions in the ribosomal L3 protein (*rplC* gene) of the PTC (Table 4). In particular, Gly152Asp in the L3 protein is the only mechanism of resistance to LZD found in isolate M32, the strain that expresses the lowest MIC (8 μg/ml) against LZD and that shows a different profile from the others by PFGE (see below). We also note that Gly152Asp was previously observed in laboratory-derived LRSA strains (23). Recently, the role of L3 in the setting of clinical LRSA was reported by Locke et al., who described two clinical isolates, one with a deletion of Ser145 and the other with an Ala157Arg substitution (22). Therefore, our findings of two additional *S. aureus* clinical strains possessing this novel mechanism of resistance emphasize the importance of screening not only the domain V of the 23S rRNA genes but also the molecular composition of the PTC.

Genotyping. As shown in Fig. 2A, all LRSA isolates tested showed >95% genetic relatedness by rep-PCR. PFGE analysis indicates that seven of the eight strains are indistinguishable ($n = 3$) or closely related ($n = 4$), whereas the one isolate (M32) that does not possess mutations in the domain V of the 23S rRNA copies is only possibly related to the others (Fig. 2B). PCR/ESI-MS shows that all eight LRSA isolates were classified into PCR/ESI-MS type 2, which includes clonal complex 5 (CC5), the USA 100 or USA 800 type by PFGE, and ST5, ST225, or ST371 by MLST typing (17). All isolates were

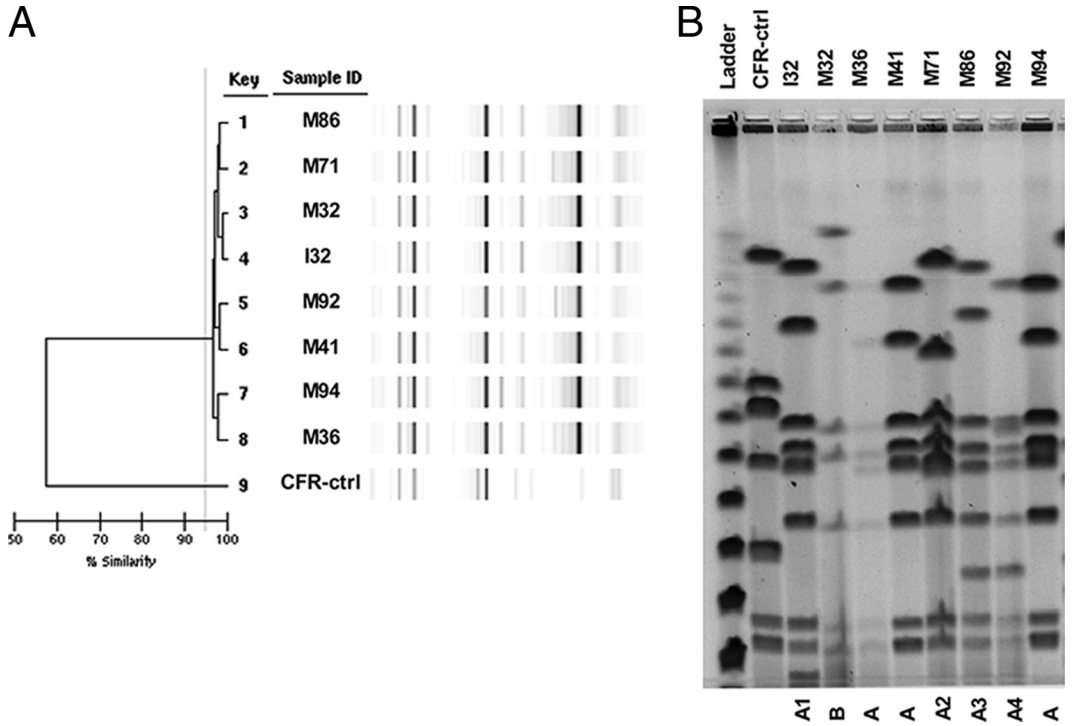

FIG. 2. Genotyping analysis of the eight LRSA isolates responsible for RTIs in CF patients. Five isolates (i.e., M36, I32, M41, M94, and M86) were collected from 2000 to 2006, whereas three (i.e., M92, M32, and M71) were collected in 2009 but were not included in the clinical statistical analysis covering the 7-year period (2000 to 2006). One LRSA isolate (CFR-ctrl) possessing the *cfr* gene was used as a control. (A) Rep-PCR results interpreted with the DiversiLab web-based software using the Kullback-Leibler method shows that all LRSA isolates tested possess genetic relatedness of ≥95%. (B) PFGE profiles with analysis of differences in the band patterns determined by visual inspection and interpreted following the recommendations of Tenover et al. (42) show that three LRSA isolates are indistinguishable (lanes A), four are closely related (lanes A1 to A4), and one (lane B) is possibly related to the others. The LRSA control strain with *cfr*-mediated resistance is not genetically related by rep-PCR and PFGE to the eight LRSA isolates found in Cleveland.

also confirmed to be *S. aureus* and were *mecA* positive, whereas genes conferring mupirocin resistance and production of the toxins PVL, γ-hemolysin, and TSST-1 were not detected (data not shown). Standard MLST identified seven isolates to be ST5, whereas strain M36 had a new MLST (ST1788) which was a single-locus variant of ST5. The analysis of the *spa* gene showed that the LRSA isolates were included in *spa* types with identical or highly similar repeat profiles, with strain M32 possessing a new *spa* type that was designated t7327 (Table 5).

S. aureus ST5 is a less pathogenic lineage and is a common health care-associated MRSA genotype reported in the United States and other countries (6). However, ST5 and ST1788 have not previously been reported among CF patients. In contrast, MRSA isolates of ST36 and ST228 have been detected in CF

TABLE 5. Comparison of MLST results obtained with two methodologies and *spa* typing

LRSA isolate	PFGE type	MLST		*spa*	
		PCR/ESI-MS[a]	Standard analysis[b]	Type	Repeats
M36	A	CC5, USA100/800, ST5/ST225/ST371	ST1788	t1062	26-23-17-34-02-17-12-17-16
I32	A1	CC5, USA100/800, ST5/ST225/ST371	ST5	t2	26-23-17-34-17-20-17-12-17-16
M41	A	CC5, USA100/800, ST5/ST225/ST371	ST5	t2	26-23-17-34-17-20-17-12-17-16
M94	A	CC5, USA100/800, ST5/ST225/ST371	ST5	t2051	26-23-17-34-17-20-16-12-17-16
M86	A3	CC5, USA100/800, ST5/ST225/ST371	ST5	t105	26-23-17-34-17-20-17-17-16
M92[c]	A4	CC5, USA100/800, ST5/ST225/ST371	ST5	t45	26-17-20-17-12-17-16
M32[c]	B	CC5, USA100/800, ST5/ST225/ST371	ST5	t7327	26-23-23-17-20-17-12-16
M71[c]	A2	CC5, USA100/800, ST5/ST225/ST371	ST5	t2115	26-23-17-13-17-20-17-17-16

[a] All isolates were also confirmed to be *S. aureus* and were *mecA* positive, whereas genes conferring mupirocin resistance and production of toxins PVL, γ-hemolysin, and TSST-1 were not detected. PCR/ESI-MS was performed on a T5000 platform.

[b] Allelic profile ST5 (*arcC*, allele 1; *aroE*, allele 4; *glpF*, allele 1; *gmK*, allele 4; *pta*, allele 12; *tpi*, allele 1; *yqiL*, allele 10) and ST1788 (*arcC*, allele 1; *aroE*, allele 4; *glpF*, allele 87; *gmK*, allele 4; *pta*, allele 12; *tpi*, allele 1; *yqiL*, allele 10).

[c] These isolates were collected in 2009 from CF patients who were not included in the clinical statistical analysis covering the 7-year period (2000 to 2006).

patients from Europe (19, 27). This suggests that there is significant heterogeneity among *S. aureus* strain types infecting CF patients and mandates an in-depth study of this population in order to better understand the transmission dynamics of these life-threatening pathogens.

Since at least seven of the LRSA isolates in our study show a high level of genetic similarity using several different genotyping methods, we speculate that these strains may have a common LRSA ancestor with at least one domain V 23S rRNA copy carrying the G2576T mutation. Under the specific selective pressure of the administered LZD regimens, this ancestor may have arisen from an ST5 MRSA strain that is probably widespread among the CF patients at our institution. The antibiotic pressure also supported the homologous recombination of other copies of the wild-type *rrn* genes with those that are mutated (45), producing the different numbers and locations of the G2576T mutation observed in the LRSA strains (Table 4). Unfortunately, this hypothesis cannot be supported by the analysis of the LSSA isolates from CF patients who subsequently developed infection due to LRSA because these isolates had not been stored for further analyses.

Conclusions. The emergence of LZD resistance in *S. aureus* represents an extreme challenge not only to the treatment of RTIs in patients with CF but also to the management of patients treated with prolonged courses of oral therapy with this drug. Although reports of LZD resistance are still uncommon (20, 35), we highlight the emergence of LRSA in CF patients at our institution, with more than 10% of patients treated with LZD developing LRSA infection. Therefore, special attention to dosing, duration of therapy, and transmission must be exercised with these patients. A critical reevaluation of the appropriate LZD dosages able to achieve an adequate PK/PD target in this population should also be done. Moreover, the genetic and molecular background of resistance from which this phenotype emerged among isolates of the most common health care-associated *S. aureus* clonal complex (i.e., CC5) in the United States suggests that screening for the emergence of LZD resistance in MRSA isolates should be undertaken during courses of therapy. Further studies are needed to continue to define the clinical and molecular epidemiology of resistance to a last-line agent against infections due to *S. aureus*.

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